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Review

The neural crest is a powerful regulator of pre-otic brain development

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ABSTRACT

The role of the neural crest (NC) in the construction of the vertebrate head was demonstrated when cell tracing techniques became available to follow the cells exiting from the cephalic neural folds in embryos of various vertebrate species. Experiments carried out in the avian embryo, using the quail/chick chimera system, were critical in showing that the entire facial skeleton and most of the skull (except for he occipital region) were derived from the NC domain of the posterior diencephalon, mesencephalon and rhombomeres 1 and 2 (r1, r2). This region of the NC was designated FSNC (for Facial Skeletogenic NC). One characteristic of this part of the head including the neural anlage is that it remains free of expression of the homeotic genes of the Hox-clusters. In an attempt to see whether this rostral Hox-negative domain of the NC has a specific role in the development of the skeleton, we have surgically removed it in chick embryos at 5–6 somite stages (5–6 ss). The operated embryos showed a complete absence of facial and skull cartilages and bones showing that the Hox expressing domain of the NC caudally located to the excision did not regenerate to replace the anterior NC. In addition to the deficit in skeletal structures, the operated embryos exhibited severe brain defects resulting in anencephaly. Experiments described here have shown that the neural crest cells regulate the amount of Fgf8 produced by the two brain organizers, the Anterior Neural Ridge (ANR) and the isthmus. This regulation is exerted via the secretion of anti-BMP signaling molecules (e.g. Gremlin and Noggin), which decrease BMP production hence enhancing the amount of Fgf8 synthesized in the ANR (also called "Prosencephalic organizer") and the isthmus. In addition to its role in building up the face and skull, the NC is therefore an important signaling center for brain development.

Introduction

The neural crest (NC) plays a critical role in the construction of the vertebrate head. This was first demonstrated by cell marking techniques that made it possible to follow migrating neural crest cells as they emerged from the cephalic neural folds in embryos of several vertebrate species. For example, experiments performed in the avian embryo, using the quail/chick chimera system, were demonstrated that the entire facial skeleton and most of the skull (except for the occipital region) are derived from the NC domain of the posterior diencephalon, mesencephalon and rhombomeres 1 and 2 (r1, r2; for reviews, see Le Douarin, 1982; Le Douarin and Kalcheim, 1999, and references therein). This discovery of this region, designated Facial Skeletogenic neural crest (FSNC), inspired Carl Gans and Glenn Northcutt to propose that the NC was an important asset in the evolution of vertebrates because it facilitated the development of a "New Head" that does not exist in the Prochordates (i.e. Cephalochordates and Urochordates).

Vertebrates are thought to have evolved from ancestors that resembled the extant Cephalochordate, the Amphioxus, whose body plan is similar to that of vertebrates. Chordates are characterized by a dorsal neural tube, a notochord and a digestive tract endowed with gill slits. They have segmented dorsal muscles, homologous to the striated muscles generated by the somitic myotomes in vertebrates. Amphioxus, however are devoid of skeletal tissues and they do not have a head. Their neural tube is slightly enlarged rostrally, thus representing a rudimentary encephalic vesicle considered as homologue to the vertebrate diencephalon (Lacalli et al., 1994). The Amphioxus embryo does not have a NC (Meulemans and Bronner-Fraser, 2004). In contrast, extinct animals such as *Haikouella* found in early Cambrian strata of Yunan (South-western China) appear to have neural crest, and, accordingly have been designated *Precraniates*, *crest animals* or *Cristozoa* (Chen, 2008; Holland and Chen, 2001). Precraniates do not have a skull but have muscular ventilation with gill-bearing and jointed branchial arches, paired head sensorial organs (eyes, nostrils) and a brain significantly larger and more complex than that of Amphioxus, which contains two morphological parts homologous to a forebrain and hindbrain, but no clear midbrain. No cerebral hemisphere structures related to the telencephalon are seen, but paired small bulges have been interpreted as olfactory nerves or bulbs.

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Two lateral eyes are connected to a diencephalon lying at its posterior end; based on gene expression pattern, the posterior part of the brain is located from the boundary of somites 2 and 3 down to that of somites 4 and 5 (Holland et al., 1992). These findings support the notion that the presence of a NC in Precraniates, which can be considered as early vertebrate, was accompanied by the elaboration of the rudimentary encephalic vesicle seen in "crestless" Cephalochordates.

It is interesting to note that the presence of the neural crest in Precraniates correlated with enhanced brain development. Thus, these observations support the ideas developed by Gans and Northcutt (1983) that the NC might have been a "promoter" of brain development in the vertebrate phylum.

Here, we review a series of experimental investigations, carried out over three decades, to study the development of the head in avian embryo. They first analyzed the origin of the NC cells, which yield the skeletal structures of the head. More recently, they have led us to demonstrate that the skeletogenic cranial neural crest (CNC) has an early role as a signaling center that controls the amount of Fgf8 produced by the two brain organizers that are active early in encephalic neurogenesis: the Anterior Neural Ridge and the midbrain–hindbrain boundary (or Isthmus), as well as the ectoderm of the first branchial arch (BA1) which is at the site of origin of the jaw.

Constructing the fate map of the anterior neural plate

In a series of experiments in the 1980s, Couly and Le Douarin constructed a fate map of the neural fold of the early avian embryo using the quail/chick chimera. Interestingly, the rostral-most part of the neural fold (NF) does not participate in NC cell emigration but fulfilled different fates, such as forming the olfactory placode. The part of the NC which participates in the formation of the skull and facial skeleton was restricted to the NF lying between the level of the epiphysis anlage and the boundary situated between rhombomeres 2 and 3 (Fig. 1; Couly and Le Douarin, 1985; 1987; Couly et al., 1996; Köntges and Lumsden, 1996).

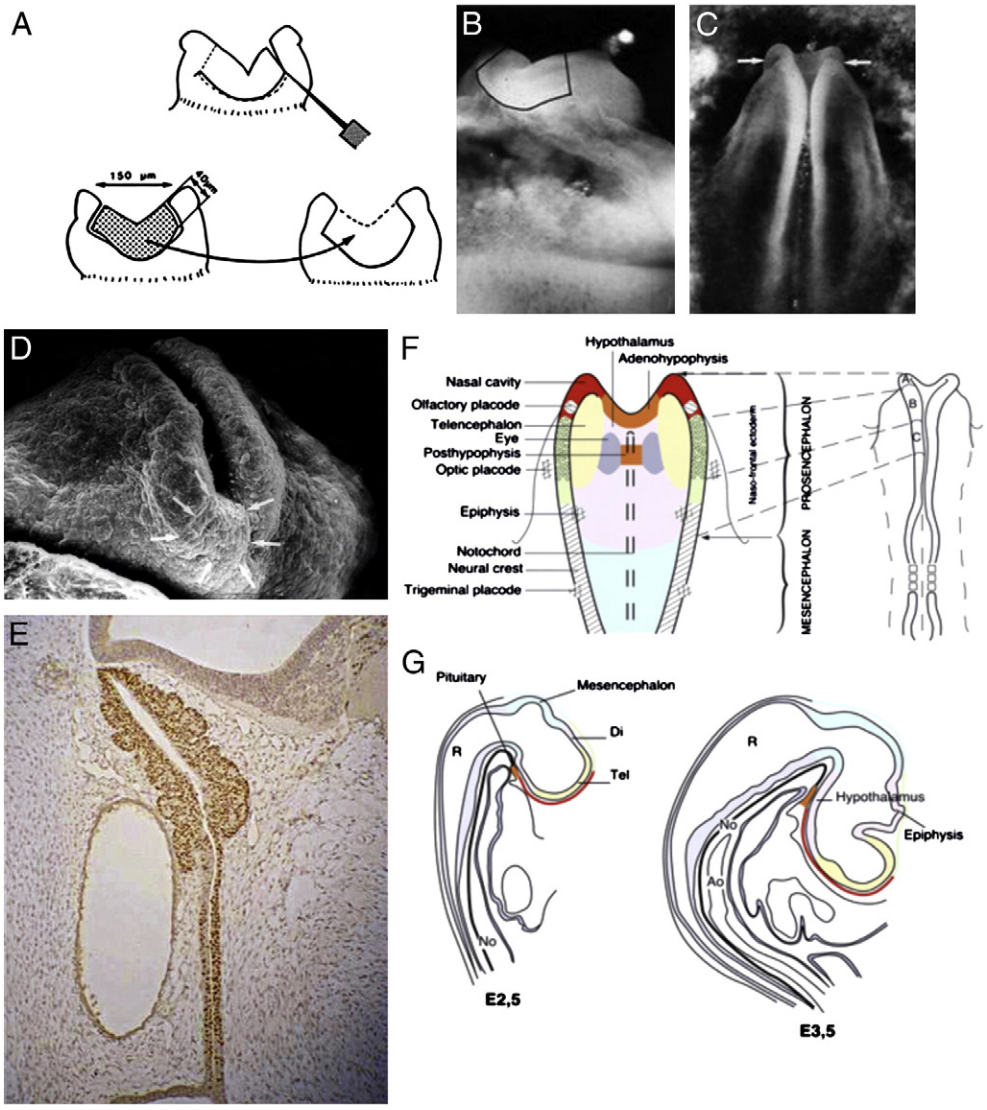

Fig. 1. Construction of the fate map of the rostral neural plate of the avian embryo. (A) Experimental design consisting in the substitution of a fragment (anterior) of the NF of the neural anlage of a 3–4 ss chick embryo by its quail counterpart. (B) The fragment of NF is labeled on the embryo. (C) The graft of the quail NF can be seen immediately after the operation. (D) View of the graft a few hours after grafting in SEM. (E) Sagittal section of the chick host treated with the QCPN Mab, which recognizes a quail specific antigenic determinant. The quail neuroepithelium that was transplanted has produced Rathke's pouch. (F) Similar experiments involving various regions of the early neural plate led to the identification of areas fated to give rise to definite regions of the brain and sense organs as indicated on the anterior neural plate before neural tube closure. (G) Two steps of encephalogenesis are represented on sagittal sections of the head showing the respective positions and fates of the areas determined at earlier stages (F). The telencephalon is formed by the lateral-most regions of the anterior neural plate, which develop between the two optic vesicles and in front of the tip of the notochord.
Reprinted, with permission, from Le Douarin and Kalcheim, 1999. Cambridge University Press.

The head skeleton is a "product" of the early neural primordium

The contribution of the neural crest (NC) to the facial skeleton was proposed for the first time by Julia Platt (1893). The NC had been discovered only two decades before by Wilhelm His (1868) and described as ectodermal cells emigrating from the lateral borders of the neural plate when they fuse dorsally during neural tube closure. Platt's observations thus implied that the mesodermal germ layer is not the exclusive source of mesenchymal cells and skeletal elements as universally accepted at that time according to the powerful *germ layer theory* of von Baer. The fact that skeletal cell types could be derived from the ectodermal germ layer seemed unacceptable for most histologists and embryologists. During the first half of the XXth century however, the findings of Julia Platt were fully confirmed. However, the NC origin of cartilage and bone remained very controversial. As a consequence, there was a long gap separating Platt's work from the remarkable series of seminal experiments carried out by Hörstadius and Sellman (for a review, see Hörstadius, 1950), on *Amblystoma jeffersonianum*, that definitively demonstrated the skeletogenic capacities of NC cells. Despite these important findings, only in the 1970–1980s was it firmly established that the entire facial skeleton together with a large part of the skull originates from the cephalic NC, not only in lower (Amphibians and Fishes), but also in amniote vertebrates. This finding relied upon the use of cell markers: the labeling of the NC cells with tritiated thymidine in the Amphibian embryos (Chibon, 1966) and the construction of chimeras between quail and chick embryos whose respective cells could be recognized either by the structure of their interphase nuclei or through the use of species-specific monoclonal antibodies (Mab) (for reviews, see Le Douarin, 1982; Le Douarin and Kalcheim, 1999). A large series of investigations clearly demonstrated that the complete facial skeleton, the frontal, parietal, squamosal bones and the part of the otic capsule were of NC origin (Couly et al., 1993; Johnston et al., 1974; Le Lièvre, 1974, 1978; Noden, 1978). Only after the rediscovery of hindbrain segmentation into rhombomeres by Andrew Lumsden, Roger Keynes and coworkers (Lumsden and Keynes, 1989; Lumsden et al., 1991) was the precise origin of the NCC participating in head and face skeleton carefully understood (Couly et al., 1996; Köntges and Lumsden, 1996) (Fig. 2A–C).

Cranial neural crest cells contribute to different facial structures depending upon their site of origin. The frontal bone, covering the forebrain, is formed by NC arising from the posterior diencephalon and anterior mesencephalon, which was found, in birds to form the parietal bone (Couly et al., 1993). The first branchial arch, BA1, which gives rise to the lower jaw, is colonized by NC cells originating from the posterior mesencephalon and the two first rhombomeres (r1–r2), whereas BA2 receives most of its NC from r4 (and to a lesser extent from r3 and r5). The third rhombomere contributes a small number of cells to both BA1 and BA2. The posterior rhombomeres provide cells which form hyoid cartilage also contribute to the cono-truncus of the heart (designated as "the cardiac crest" according to Kirby et al. (1983, 1985). In addition to the skull and facial skeleton, the cephalic NC gives rise to connective tissues involved in facial striated muscle histogenesis as well as the meninges of the forebrain and the dermis of the face and ventral part of the neck (Etchevers et al., 1999; 2001; Le Lièvre and Le Douarin, 1975).

The mesodermal contribution to head skeleton includes the occipital bones and part of the otic capsule. It is noteworthy that the mesenchymal cells form endothelial cells lining the blood vessel throughout the body. In the head, many of these mesenchymal cells are derived from the neural crest. As a result, in this part of the body the pericytes and the musculo-connective components of the blood vessels are of ectodermal origin, via the NC.

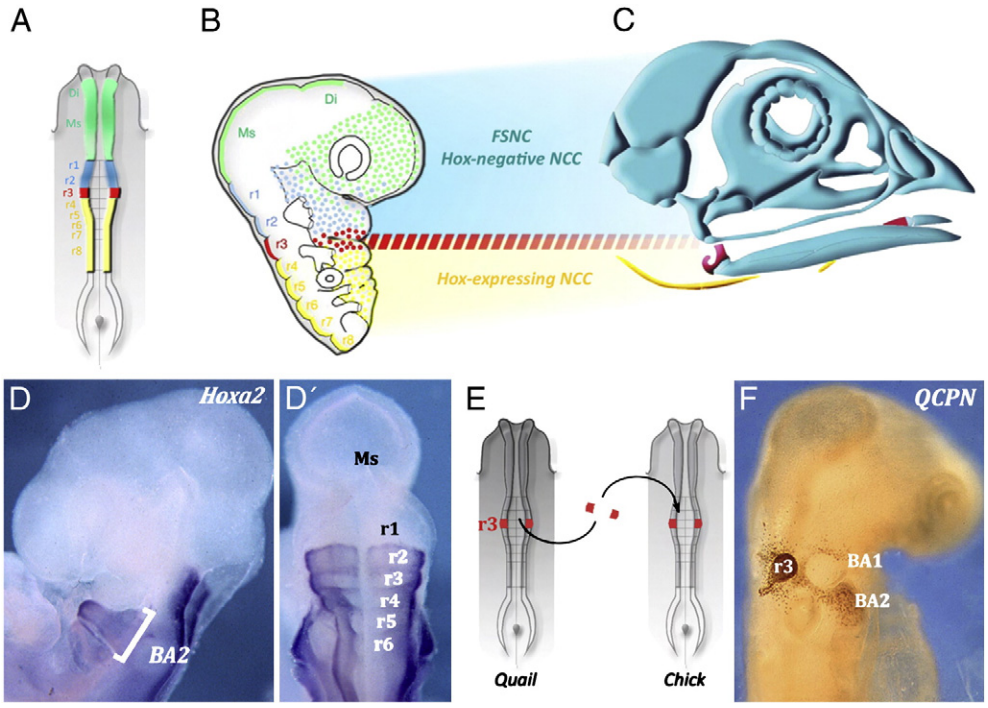

Fig. 2. Contribution of the CNC to head development. (A) The color-coded representation of the cephalic neural fold in a 5-ss embryo: the di- and mesencephalic NC appears in green, r1–r2 NC in cyan, to r3 NC in red, and to r4–r8 NC in yellow. (B) Colonization of the facial processes and BAs by the CNCC according to their level of origin. (C) The skull vault, nasal and orbital capsules, jaws and entoglossum are derived from the Hox-negative FSNC. The hypobranchial skeleton corresponding to the posterior part of the hyoid bone is derived from Hox-expressing NCC (in yellow). Interposed between these two domains, r3 NCC contribute to the retro-articular and to the medial third of the basihyal (in red). (D) Accumulation of *Hoxa2* transcripts in BA2 and the more posterior BAs at E3; rostrally, the head is devoid of *Hoxa2* expression. (D′) *Hoxa2* is expressed in the hindbrain up the boundary between r2 and r1. (E) Schematic representation of r3-NC graft from quail to chick at 5 ss. (F) Twenty-four hours post-grafting, the quail CNCC – identified using the QCPN Mab – have migrated from their site of implantation and contributed mesenchymal cells to both BA1 and BA2. BA, branchial arch; Di, diencephalon; Ms, mesencephalon; r, rhombomere.

From this perspective, one can consider that *the face and most of the skull are the "product" of the neural anlage, the neural crest, which makes a critical contribution to cephalic structures.*

Different genetic patterning for head and trunk in vertebrates

In the 1980–1990s, it was observed that the genes of the four Hox-clusters, which play a key role in patterning the body, are not expressed in the cephalic region of the vertebrate embryo. With respect to the neural crest, this means that cells contributing to Facial Skeletal neural crest (FSNC) lack Hox gene expression. In other words, the NCC colonizing the naso-frontal buds and the maxillo-mandibular arch (BA1) do not express any gene of the four Hox-clusters. In the neural tube the rostral-most level of expression of these genes, corresponding to *Hoxa*, occurs at the boundary between r1 and r2 (Fig. 2D). Rhombomere 3 (r3) forms an intermediate zone between a rostral Hox-negative and a caudal Hox-positive domain of the NC (Fig. 2E, F).

Hox gene expression by the FSNC prevents the development of the head skeleton

This raised the intriguing question of whether Hox gene expression might be compatible with the development of the skeletal structures of the face and skull, which normally derive from the Hox-negative domain of the NC.

To test this possibility, *Hoxa2*, *-a3*, *-b4* were misexpressed in the cephalic NC prior to their emigration, by means of electroporation of viral constructs encoding *Hox* proteins into the NF. The results showed that, although the neural derivatives of the CNC developed normally, no skeletal derivatives differentiated in the treated embryos (Fig. 3A–L). *Hoxa3* and *Hoxb4* respectively perturbed the development of skeletal structures in a complementary manner. The combined addition of *Hoxa3* and *Hoxb4* cDNAs reproduced the effect of *Hoxa2* alone (Creuzet et al., 2002).

To further examine the importance of the neural crest in brain development, the Hox negative neural folds were surgically ablated just above rhombomere 3. The phenotype of the embryos in which the FSNC had been removed was very striking: no facial structures developed, the facial and naso-frontal buds remained empty of mesenchymal cells in the absence of the Hox-negative domain of the NF. Thus, remaining Hox-positive cells were unable to replace the missing territory to construct facial and skull structures. Similarly, removing the Hox-negative neural crest and replacing it with the equivalent length of Hox-positive NF (from r4 to r8; see Fig. 3M–P) failed to rescue head and face development. Therefore, the Hox-positive domain of the cephalic NC can neither regenerate to replace the rostral one nor provide the developing head with NCC competent to differentiate into skeletal tissue when placed in the Hox-negative environment of the head.

Strikingly, even small pieces of Hox-negative NC retain the capacity to regenerate the removed FSNC. After ablation of this territory, fragments from stage-matched quail embryos of less than a third of the length of the removed region (Fig. 3Q) were able to completely rescue the cephalic region of the operated embryos (Fig. 3R). These results demonstrate that the Hox-negative domain

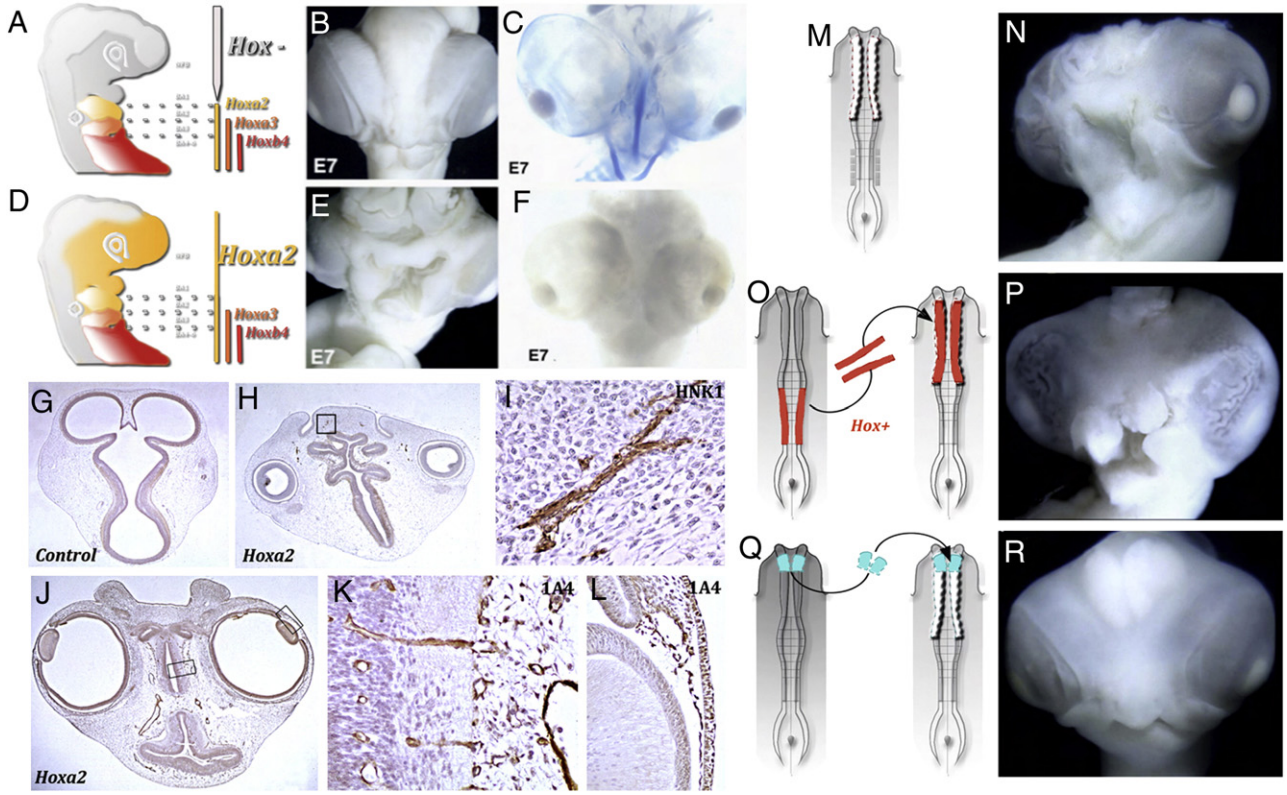

Fig. 3. Detrimental effect of Hox gene expression on head development. (A–C) In normal conditions, when the anterior domain of the NC is Hox-free (A), the fronto-nasal and the maxillo-mandibular structures develop at E7 (B); Alcian blue staining showing the nasal skeleton and mandibles (C). (D–F) When *Hoxa2* is misexpressed in the FSNC, selectively (D), its expression totally ruins the facial development, resulting in anencephaly (E); skeletal differentiation is blocked (F). (G) Brain development in E4 control embryo. (H) When the FSNC is transfected with *Hoxa2* constructs, forebrain vesicles are collapsed. However, FSNC-derived glial cells can develop in peripheral nerves as evidenced by HNK1 mAb (I). At the diencephalic level (J), *Hoxa2*-transfected FSNC cells give rise to pericytes (that accumulate a-smooth actin, detected by 1A4 Ab) to the forebrain vasculature (K) and differentiate into ciliary muscles and corneal endothelium (L). (M) Bilateral extirpation of the FSNC at 5 ss leads to the absence of facial structures and to anencephaly (N). (O) Bilateral substitution of quail r4–r8 NCC to the FSNC results in the complete absence of upper face and lower jaw (P). (Q) In embryos after excision of the FSNC, a bilateral graft of the posterior diencephalic NC is sufficient to restore a normal head development (R).
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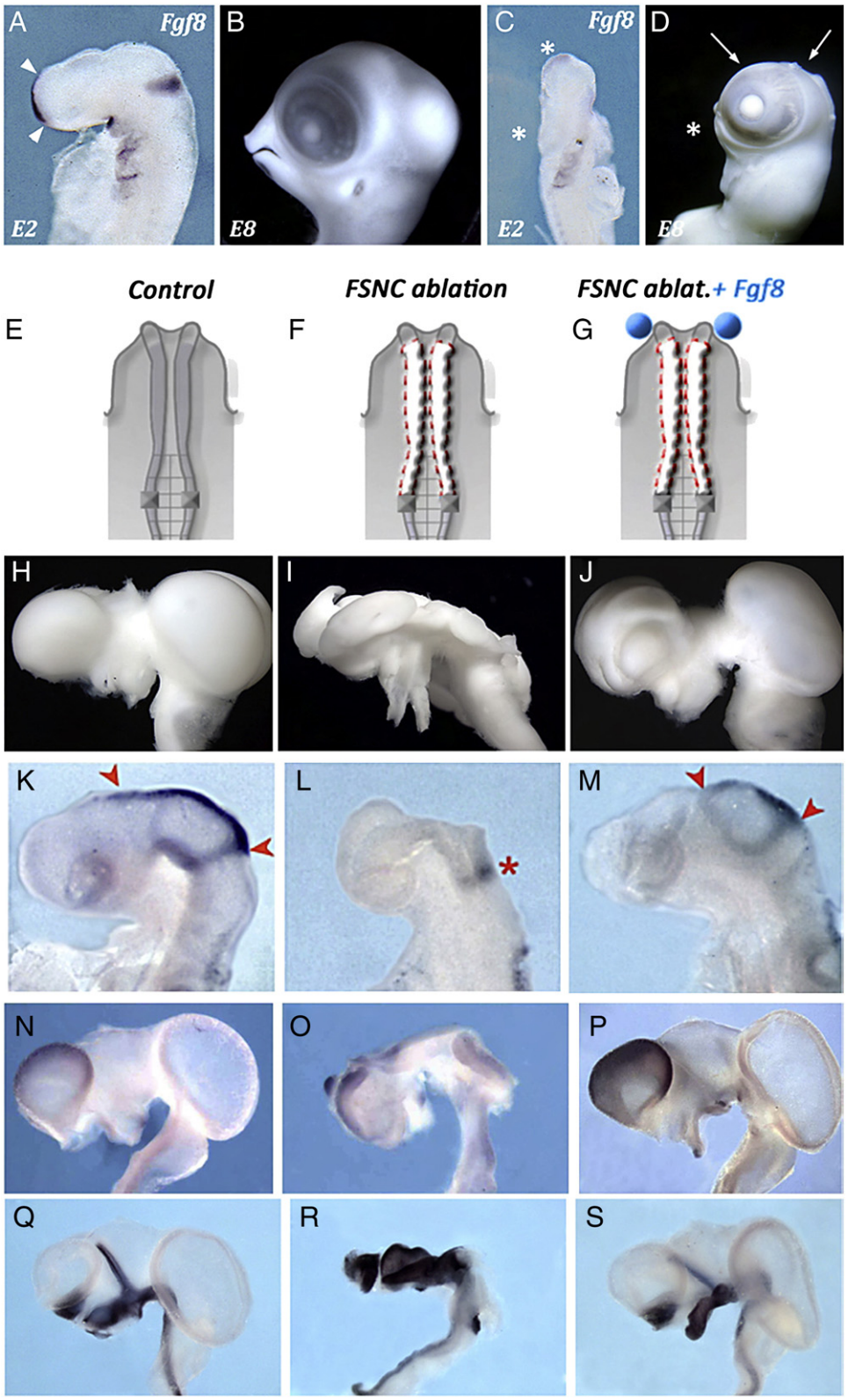


Fig. 4. Absence of FSNC alters Fgf8 expression and cephalic development. (A, C) *Fgf8* expression in 24 ss control (A, arrowheads) and FSNC-deprived (C) embryos showing the loss of *Fgf8* expression in ANR and BA1 (star) after FSNC ablation at 5 ss. (B, D) Morphology of E8 control (B) and FSNC-deprived (D) embryos exhibiting the absence of facial (star) and cerebral structures (arrows). (E–S) Morphology and molecular profile of the developing brains in normal condition (E), in absence of FSNC (F), in absence of FSNC and supplementation with Fgf8 (G). (H–J) Whole-mount brain preparations dissected out from control (*H*); FSNC-deprived (*I*); and FGF8-supplemented, FSNC-deprived (*J*) embryos at E6. (*I*) In absence of FSNC, the brain is wide-open and partitions into telencephalon, diencephalon, and mesencephalon are no longer recognizable. (*J*) With Fgf8 in the ANR, the progression of NCC rostrally restores neural tube closure and brain regionalization. (K–M) At 24 ss, *Wnt1* expression in the dorsal mesencephalon and thalamus (K; arrowheads) is lost in the absence of FSNC, except at the level of the isthmus (L; star) but can be restored when Fgf8 is supplemented in ANR (M). (N–P) *Emx2* is expressed in the dorsal telencephalon and in few cells in the hypothalamus (N). *Emx2* expression is severely perturbed in FSNC-ablated embryos (O), and is restored in Fgf8-supplemented embryos (P). (Q–S) *Shh*, in normal development, is expressed in the prosencephalic basal plate, ZLI and subpallial structures (Q). In FSNC-deprived embryos (R), *Shh* transcript accumulation is expanded at the expense of the alar plate, but tends to normalize with Fgf8 supplementation (S). Creuzet et al., 2006, Proc. Natl. Acad. Sci. USA, 103, 1433–1438. © 2006, National Academy of Sciences, U.S.A.

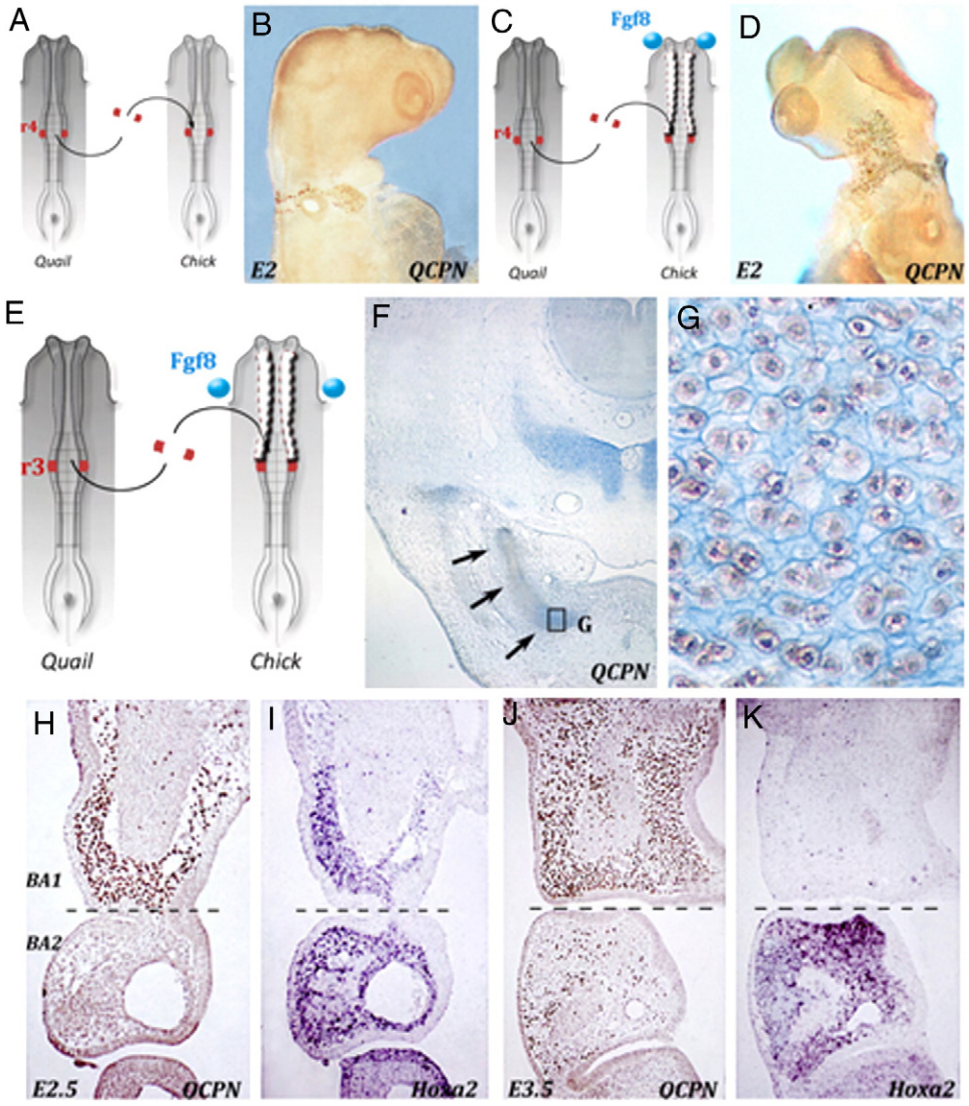


Fig. 5. Fgf8 expression is required for NCC migration. (A) Replacement of r4-NCC in normal chick embryo by its quail counterpart shows that at E2 (B), the r4-NCC populate BA2 exclusively. (C) When FSNC is removed together with r3-NC in Fgf8-treated embryo, engrafted quail r4-NCC migrate rostrally but fail to populate BA1 (D). (E) In Fgf8-treated FSNC-ablated embryos, the r3-NC is replaced by its quail counterpart. (F, G) At E6.5, r3-derived NCC can rescue facial development and give rise to the mandible (F; arrows), which is entirely made up of quail cells (G). Under these conditions, the r3-derived NCC that are populating BA1 at E2.5 (H), express *Hoxa2* (I). At E3.5, the r3-derived NCC (J) have turned to an Hox-negative status in BA1 (K), while they have maintained the expression of *Hoxa2* in BA2 (K). BA, branchial arch; r, rhombomere.
Creuzet et al., 2004, Proc. Natl. Acad. Sci. USA, 101, 4843–4847. © 2004, National Academy of Sciences, U.S.A.

of the NC behaves like a "morphogenetic field". These cells are competent to produce facial and skull structures at that stage and a smaller fraction of this field has the capacity to replace the whole in building the neural crest-derived portion face and part of the skull.

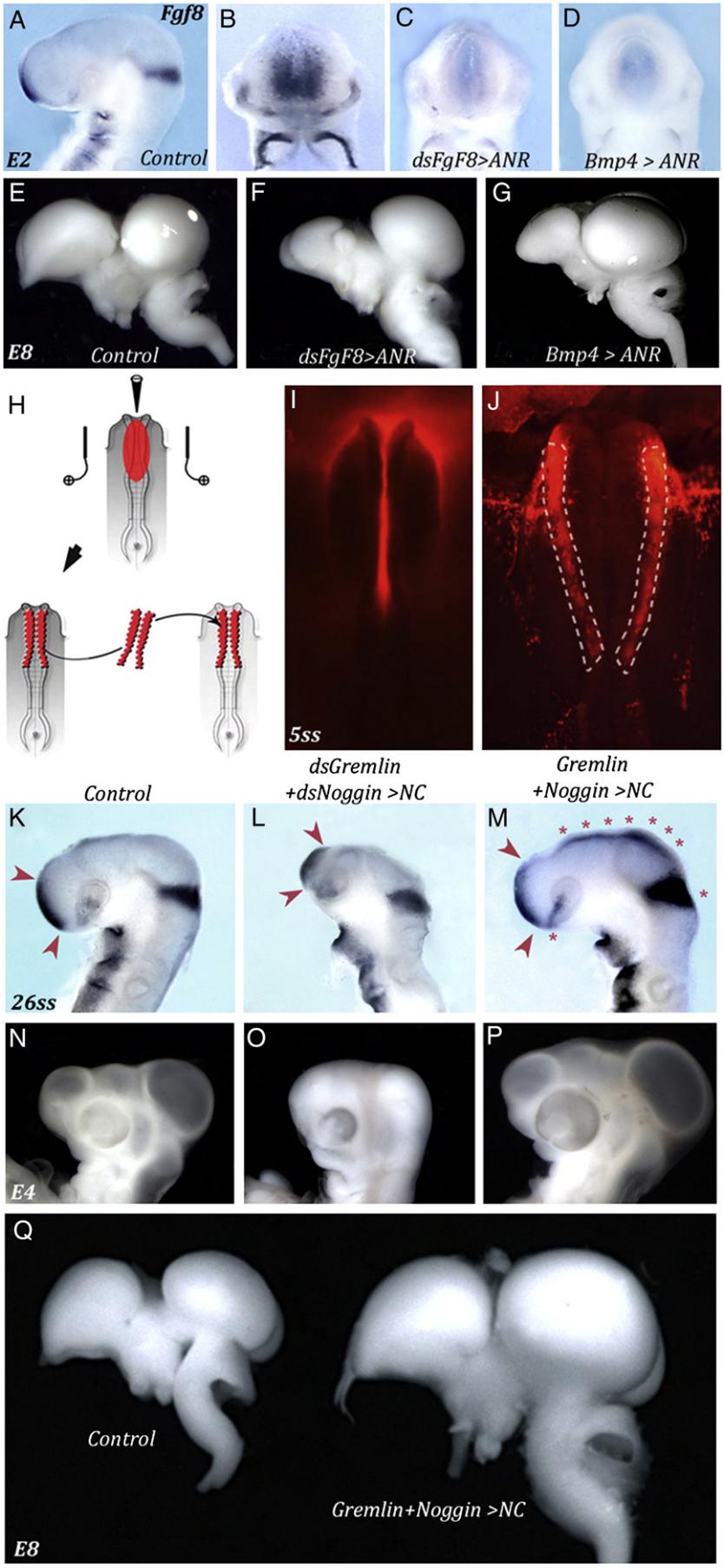
In addition to missing neural crest derivatives, the operated embryos exhibited extensive brain defects consisting of anencephaly with strong reduction of the telencephalon and of the derivatives of the anterior alar plate territories of the neural anlage, and which normally yield the thalamus and the optic *tectum*. Furthermore, normal expression of genes in the pros- and mesencephalon was either strongly diminished or totally abolished (Fig. 4). Transcripts of the genes normally expressed dorsally in 3.5 day-old embryos (i.e. 25 ss) were absent. In contrast, *Shh* whose expression area is mostly ventral (except for the *Zona Limitans Intrathalamica* – ZLI – and the Medial Ganglionic Eminence – MGE) in control embryos, exhibited a lateral extension, such that the whole neural epithelium of embryos devoid of FSNC strongly expressed this gene.

These results suggest that removal of the rostral Hox-negative domain of the NC prevents the development of the alar plates of the rostral neural anlage thus resulting in a "ventralization" of the neural structures arising from the basal plates.

The removal of the rostral Hox-negative domain of the NC depletes the secondary "brain organizers" of Fgf8

Brain development depends upon the signaling activity of the so-called "secondary brain organizers" producing Fgf8 during the early stage of neurogenesis: the isthmus, or midbrain–hindbrain boundary (MHB) which patterns the cerebellum and the optic tectum (Martinez et al., 1991) and the Anterior Neural Ridge (ANR) also designated as "telencephalic organizer" which is critical for the development of the forebrain (Houart et al., 1998; Shimamura and Rubenstein, 1997).

Following the removal of the FSNC, the level of *Fgf8* transcripts in both sites was greatly reduced (Fig. 4A–D). Moreover, the phenotype resulting from the operation could be rescued by the addition of recombinant Fgf8 soaked beads placed either laterally to the mesencephalon or on each side of the ANR at 5–6 ss (Fig. 4E–G). Not only did facial and skeletal structures develop under these conditions,


A
Fgf8
E2
Control
B
C
dsFgF8>ANR
D
Bmp4 > ANR
E
E8
Control
F
dsFgF8>ANR
G
Bmp4 > ANR
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I
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Control
dsGremlin
+dsNoggin >NC
Gremlin
+Noggin >NC
K
26ss
L
M
N
E4
O
P
Q
Control
Gremlin+Noggin >NC
E8

but the cephalic vesicles were also morphologically near normal and displayed appropriate patterns of gene expression (Creuzet et al., 2006; Fig. 4H–S).

Rescue of the FSNC removal phenotype by exogenous Fgf8 is mediated by r3-derived NCC

These results raise the intriguing question of the origin of the NCC that gave rise to the facial skeleton in the FGF8-rescued embryos. To address this issue, the experiment was repeated of grafting quail r3 into embryos after ablation of the Hox-negative neural crest, with the addition of placing FGF8-soaked beads. Specifically, the experiment consisted of 1) removing the FSNC in a 5 ss chick-embryo, i.e. the NF from the level of the mid-diencephalon down to r2-included, 2) replacing the pair of rhombomeres three (r3) of this embryo by their equivalent from a stage-matched quail, and 3) placing Fgf8-soaked beads on each side of the mesencephalon of the operated embryo. The NC derived structures of the head were then characterized as being either of chick, quail or mixed origin. Interestingly, the results revealed that all the NC derivatives of the operated embryos were comprised of quail cells (Fig. 5A–G) (Creuzet et al., 2004). This very striking result demonstrated that Fgf8 is a key signaling molecule at this stage of head development.

In these experiments, the amount of Fgf8, which diffused from the beads, was sufficient to promote proliferation of the NCC exiting from r3. Normally, the r3 contribution to BA1 is small. It has been shown that this effect, results from inhibition exerted by the adjacent rhombomeres r2 and r4 (Graham et al., 1993; 1994). In the absence of the FSNC, however, the number of r3 NCC was not sufficient to populate the anterior domain of the head. Therefore, exogenous Fgf8 provided by the beads had a strong proliferative effect on r3 cells and also may have acted as a chemo-attractant promoting invasion of the whole cephalic domain of the experimental embryo (Creuzet et al., 2004).

In normal development, the NCC originating from r3 and colonizing BA1, express Hoxa2 transiently but down-regulate this gene when they reach the Hox-negative environment of BA1. In contrast the caudal stream of r3-derived cells that contributes to BA2 maintains its *Hoxa2* expression. In the experimental situation described above a large flux of r3 NCC invades the head. This raised the question of whether they would maintain their Hox-gene expression, via a "cell-community effect" (see Trainor and Krumlauf, 2000). At E2.5, the r3-derived NCC that populate BA1 express *Hoxa2*. However, one day later, there are many more cells and they have lost *Hoxa2* expression whereas r3 derived cells that colonized BA2 in the same embryos maintained it (Fig. 5H–K). This observation is consistent with the idea that facial structures can develop only from NCC that do not express Hox genes.

r3 is situated between the Hox-negative rostral and the Hox-positive caudal domains of the CNC and can therefore be considered as an intermediate zone that produces both Hox-negative and Hox-positive neural crest. In contrast, those cells emigrating from a more posterior rhombomere, like r4, normally only generate Hox-positive NC. This raises the question of whether r4 cells would respond similarly to Fgf8 following the removal of the rostral domain of the NC, including r3.

To answer this question, the above experiment was repeated by removing the FSNC including r3 in the presence of Fgf8-soaked beads. The results showed that r4 NC migrated rostrally only a short distance and were not able to colonize BA1 (Fig. 5). Moreover, they did not show the same plasticity as those of r3 and maintained their expression of *Hoxa2*. Thus, r4 NC cells were not able to rescue the phenotype of the embryos subjected to FSNC ablation even when treated with Fgf8 soaked beads. This can be attributed to the fact that the NCC arising from rhombomeres caudal to r3 express *Hox* genes.

This series of experiments demonstrate that expression of *Hox* genes in the NCC is not compatible with their differentiation into skeletal tissues (either membrane bones or cartilage). Moreover, they also show that the NC, which is responsible for producing the facial skeleton, has another very important function in vertebrate head development: it is indispensable for the development of the brain.

Regulation of Fgf8 production in the ANR, isthmus and BA1 ectoderm by the cephalic NC

Brain development is influenced by particular regions of the developing cephalic vesicles called *secondary organizers* that produce signaling molecules like Fgf8 (Rubenstein and Beachy, 1998). The first of these organizers was identified at midbrain–hindbrain boundary (MHB or isthmus) (Martinez et al., 1991). The second, the Anterior Neural Ridge (ANR), was also designated as *telencephalic organizer* because of its role on the development of the forebrain (Houart et al., 1998; Shimamura and Rubenstein, 1997). Other signaling molecules are also expressed in the developing brain including bone morphogenetic proteins (Bmps) expressed in the prechordal plate and in the dorsal prosencephalon, whereas Sonic hedgehog (Shh) production is restricted to the ventral region.

Experiments in the chick and mouse clearly have demonstrated that Bmps can inhibit *Fgf8* expression in the ANR, while Shh maintains it. This suggested that regulation of morphogenesis of the forebrain was the result of cross-regulation between Bmps, Fgfs and Shh signaling centers (Ohkubo et al., 2002). Our experiments further suggest the existence of an additional player in this complex process: the cephalic Hox-negative domain of the NC.

During early stages of neurulation, the cranial NC has been shown to express genes encoding Bmp-antagonists such as *Gremlin* and *Noggin* (Tzahor et al., 2003). This led to the intriguing hypothesis that removal of the FSNC, source of these signaling molecules, might be responsible for an increase in the effect of Bmps on the production of Fgf8 by the ANR, the isthmus and BA ectoderm. As a first step in testing this hypothesis, Fgf8 production by the ANR was decreased by electroporating dsRNA against *Fgf8* at 5–6 ss. Second, synthesis of Bmp4 in the prosencephalic area was increased by the electroporation of RCAS-Bmp4. Both approaches yielded similar results. The amount of *Fgf8* transcripts in the ANR was strongly reduced in both cases at 24–26 ss, i.e., when it is normally at its maximum (Fig. 6A–D). Thirty hours after the injection, the forebrain was found to be hypomorphic with a disorganization of inter-hemispheric structures, absence of olfactory bulb and nerve and the atrophy of the choroid plexuses (Fig. 6E–G).

To demonstrate interaction between the NC and the ANR during early neurogenesis, the Bmp inhibitors *Gremlin* and *Noggin*, were

Fig. 6. Signals controlling Fgf8 expression in ANR. (A–D) Expression of Fgf8 in ANR at 24 ss (E2.5) embryo in control (A, B), Fgf8-depleted (C), Bmp4-supplemented (D) embryos. Side (A) and frontal (B) views in unoperated embryo. Following the electroporation of *Fgf8*-dsRNA (C) or *Bmp4*-RCAS (D) in the ANR in 5- to 6-ss chick embryos, *Fgf8* expression is similarly abolished in the ANR in both situations. (E–G) Morphology of E8 brains taken from control (E), *Fgf8*-dsRNA- (F) or *Bmp4*-RCAS- (G) treated embryos. Down-regulation of *Fgf8* expression in the ANR following *Fgf8*-dsRNA (*P*) and *Bmp4*-RCAS (Q) results in a dramatic reduction of the telencephalon. (H) Two-step procedure for FSNC transfection. Nucleic acids, injected in the neural groove of quail neurula, are bilaterally transfected into FSNC by electroporation before bilateral transplantation into untransfected chicken embryo. (I) Co-injection and -electroporation (J) with Rhodamine-Dextran enables visualization of the transfected NCC (J; dotted lines) before transplantation. *Fgf8* expression at E2 (K–M) and E4 morphology (N–P) of control (K and N), *Gremlin* and *Noggin*-dsRNA-treated (L and O), and *Gremlin* and *Noggin*-RCAS-treated (M and P) embryos. Silencing of Bmp-inhibitors in CNC alters *Fgf8* expression in ANR (L; arrowheads). Inversely, *Gremlin* and *Noggin* over-expression in CNC increases the expression domain of *FgF8* in the ANR (M; arrowheads) and isthmus, and induces transcript accumulation along the dorsal midline (M; stars). Normal morphology of E4 control embryo (N). (O) Silencing experiment results in underdeveloped cephalic vesicles and eyes. (L) Over-expression of *Gremlin* and *Noggin* in CNC causes the dorsal expansion of brain development (P). Whole-mount preparations of E8 pre-otic brains showing (left) the normal brain development, and (right) the hypertrophied morphology resulting from *Gremlin* and *Noggin* over-expression in FSNC.
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either down- or up-regulated in the FSNC through an experimental design depicted in Fig. 6H–J. A strong reduction of the dorsal and anterior growth of the pro- and mesencephalic alar-plates was observed after concomitant down-regulation of both Bmp inhibitors (Fig. 6K, L, N, O). In contrast, co-electroporation of *Gremlin*- and *Noggin*-RCAS considerably increased the amount of *Fgf8* transcripts in the ANR and the isthmus (Fig. 6M). At E4, the cephalic and optic vesicles were significantly enlarged, and, later the entire brain was found to be hypertrophic (Fig. 6P,Q) (Creuzet, 2009).

Concluding remarks

Taken together, these experiments show that the cephalic NC exerts a powerful effect on the development of the brain and face by regulating the amount of *Fgf8* produced by the ANR, the isthmus and branchial arch ectoderm. Fgf8 is necessary for the growth of the lateral-most areas of the anterior neural plate that are the site of origin of the telencephalon, the thalamus and the optic tectum. This regulation is mediated by the production of Bmp-antagonists produced by the NC cells while they are still in the NF throughout their migration phase as they envelop the developing brain and colonize the facial buds. Our experiments also point to the role of Fgf8 in the survival and proliferation of the NCC themselves. In our experimental conditions, Fgf8 was shown to exert such a vigorous proliferative effect upon CNCC that cells exiting from one single pair of rhombomeres (r3) were sufficient to replace the whole FSNC to produce nearly normal development of the brain and head skeleton.

These experiments show that neural crest cells primarily influence the development of more evolutionary recent parts of the brain: the telencephalon and the dorsal-most regions of the di- and mesencephalon, thalamus and optic tectum. Moreover, removal of the FSNC leads to ventralization of the encephalon evidenced by means of the lateral expansion of the *Shh* expression domain. These observations support the idea that the cephalic NC of the vertebrate embryo is a novel brain organizer operating as a regulator of the ANR and the isthmus. Moreover, Fgf8 expression by the BA ectoderm is abolished after removal of the CNC, hence precluding facial development. This observation further reinforces the notion that Brain and Face constitute a "developmental unit" under the control of the vertebrate innovation: the neural crest.

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References

Chen, J.-Y., 2008. Early crest animals and the insight they provide into the evolutionary origin of craniates. Genesis 46, 623–639.
Chibon, P., 1966. Analyse expérimentale de la regionalization et des capacities morphogénétiques de la crête neural chez l'Amphibien Urodèle Pleurodeles waltlii Michah. Mem. Soc. Fr. Zool. 36, 1–107.
Couly, G.F., Le Douarin, N.M., 1985. Mapping of the early neural primordium in quail-chick chimeras: I. Developmental relationships between placodes, facial ectoderm, and prosencephalon. Dev. Biol. 110, 422–439.
Couly, G.F., Le Douarin, N.M., 1987. Mapping of the early neural primordium in quail-chick chimeras: II. The prosencephalic neural plate and neural folds: implications for the genesis of cephalic human congenital abnormalities. Dev. Biol. 120, 198–214.
Couly, G., Coltey, P., Le Douarin, N.M., 1993. The triple origin of skull in higher vertebrates: a study in quail-chick chimeras. Development 117, 409–429.
Couly, G., Grapin-Botton, A., Coltey, P. Le, Douarin, N.M., 1996. The regeneration of the cephalic neural crest, a problem revisited: the regenerating cells originate from the contralateral or from the anterior and posterior neural fold. Development 122, 3393–3407.
Creuzet, S.E., 2009. Regulation of pre-otic brain development by the cephalic neural crest. Proc. Natl. Acad. Sci. U. S. A. 106, 15774–15779.
Creuzet, S., Couly, G., Bennaceur, S., Vincent, C., Le Douarin, N.M., 2002. Negative effect of Hox gene expression on the development of the neural crest-derived facial skeleton. Development 129, 4301–4313.
Creuzet, S., Schuler, B., Couly, G., Le Douarin, N.M., 2004. Reciprocal relationships between Fgf8 and neural crest cells in development. Proc. Natl. Acad. Sci. U. S. A. 101, 4843–4847.
Creuzet, S.E., Martinez, S., Le Douarin, N.M., 2006. The cephalic neural crest exerts a critical effect on forebrain and midbrain development. Proc. Natl. Acad. Sci. U. S. A. 103, 1433–1438.
Etchevers, H.C., Couly, G.F., Vincent, C., Le Douarin, N.M., 1999. Anterior cephalic neural crest is required for forebrain viability. Development 126, 3533–3543.
Etchevers, H.C., Vincent, C., Le Douarin, N.M., Couly, G.F., 2001. The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain. Development 128, 1059–1068.
Gans, C., Northcutt, R.G., 1983. Neural crest and the origin of vertebrates. A new head. Science 20, 268–274.
Graham, A., Heyman, I., Lumsden, A., 1993. Even-numbered rhombomeres control the apoptotic elimination of neural crest cells from odd-numbered rhombomeres in the chick. Development 119, 233–245.
Graham, A., Francis-West, F., Brickell, P., Lumsden, A., 1994. The signaling molecule BMP4 mediates apoptosis in the rhombencephalic neural crest. Nature 372, 684–686.
His, W., 1868. Untersuchung über die ersteanlage des Wirbeltierleibes. Die erste entwickling des Hühnchens. Vogel, Leipzig.
Holland, N.D., Chen, J.-Y., 2001. Origin and early evolution of the vertebrates: new insights from advances in molecular biology, anatomy and paleontology. Bioessays 23, 142–151.
Holland, P.W., Holland, L.Z., Williams, N.A., Holland, N.D., 1992. An amphioxus homeobox gene: sequence, conservation, spatial expression during development and insight into vertebrate evolution. Development 116, 653–661.
Hörstadius, S., 1950. The Neural Crest: Its Properties and Derivatives in the Light of Experimental Research. Oxford University Press, London.
Houart, C., Westerfield, M., Wilson, S.W., 1998. A small population of anterior cells patern the forebrain during zebrafish gastrulation. Nature 391, 788–792.
Johnston, M.C., Bhakdinaronk, A., Reid, Y.C., 1974. In: Bosma, J.F. (Ed.), Oral Sensation and Perception: Development in the Fetus and Infant. U.S. Govt. Printing Office, Washington.
Kirby, M.L., Gale, T.F., Stewart, D.E., 1983. Neural crest cells contribute to normal aorticopulmonary septation. Science 220, 1059–1061.
Kirby, M.L., Turnage, K.L., Hayes, B.M., 1985. Characterization of conotruncal malformations following ablation of "cardiac" neural crest. Anat. Rec. 213, 87–93.
Köntges, G., Lumsden, A., 1996. Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. Development 122, 3229–3242.
Lacalli, T.C., Holland, N.D., West, J.E., 1994. Landmarks in the anterior central nervous system of amphioxus larvae. Philos. Trans. R. Soc. London 344, 165–185.
Le Douarin, N.M., 1982. The Neural Crest. Cambridge University Press, Cambridge.
Le Douarin, N.M., Kalcheim, C., 1999. The Neural Crest, 2nd edition. Cambridge University Press, New York, Cambridge.
Le Lièvre, C.S., 1974. Rôle des cellules mésectodermiques issues des crêtes neurales céphaliques dans la formation des arcs branchiaux et du squelette viscéral. J. Embryol. Exp. Morphol. 31, 453–477.
Le Lièvre, C.S., 1978. Participation of neural crest-derived cells in the genesis of the skull in birds. J. Embryol. Exp. Morphol. 47, 17–37.
Le Lièvre, C., Le Douarin, N., 1975. Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos. J. Embryol. Exp. Morphol. 34, 125–154.
Lumsden, A., Keynes, R., 1989. Segmental patterns of neuronal development in the chick hindbrain. Nature 337, 424–428.
Lumsden, A., Sprawson, N., Graham, A., 1991. Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo. Development 113, 1281–1291.
Martinez, S., Wassef, M., Alvarado-Mallart, R.-M., 1991. Induction of a mesencephalic phenotype in the 2-day-old chick prosencephalon in preceded by the early expression of the homeobox gene En. Neuron 6, 971–981.
Meulemans, D., Bronner-Fraser, M., 2004. Gene-regulatory interactions in neural crest evolution and development. Dev. Cell 7, 291–299.
Noden, D.M., 1978. Interactions directing the migration and cytodifferentiation of avian neural crest cells. In: Garrod, D. (Ed.), The Specificity of Embryological Interactions. Chapman and Hall, London.
Ohkubo, Y., Chiang, C., Rubenstein, J.L.R., 2002. Coordinate regulation and synergistic actions of BMP4, SHH and FGF8 in the rostral prosencephalon regulate morphogenesis of the telencephalic and optic vesicles. Neuroscience 111, 1–17.
Platt, J.B., 1893. Ectodermic origin of the cartilages of the head. Anat. Anz. 8, 506–509.
Rubenstein, J.L., Beachy, P.A., 1998. Patterning of the embryonic forebrain. Curr. Opin. Neurobiol. 8, 18–26.
Shimamura, K., Rubenstein, J.L., 1997. Inductive interactions direct early regionalization of the mouse forebrain. Development 124, 2709–2718.
Trainor, P., Krumlauf, R., 2000. Plasticity in mouse neural crest cells reveals a new patterning role for cranial mesoderm. Nat. Cell Biol. 2, 96–102.
Tzahor, E., Kempf, H., Mootoosamy, R.C., Poon, A.C., Abzhanov, A., Clifford, J., Tabin, C.J., Dietrich, S., Lassar, A.B., 2003. Antagonists of Wnt and BMP signaling promote the formation of vertebrate head muscle. Genes Dev. 17, 3087–3099.